Hippocampal Circuitry Dysfunction in the 5-HT1A Knockout Mouse

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Abstract
Anxiety disorders are the most prevalent class of mental illness, yet currently available treatments are often ineffective or inadequate, leaving many patients with lingering symptoms. The serotonin 1A receptor (1AR) has been implicated in the etiology of these disorders, which often show comorbidity with cognitive dysfunction. Mice with the 1AR genetically deleted or "knocked out" (1AKO) during a critical period in development (postnatal days 13-21) exhibit anxiety-like behavior and learning and memory deficits, and may therefore represent a useful genetic model in studying the neurobiological effects of this receptor. The hippocampus has been shown to highly express the 1AR and to be a key mediator in memory and the regulation of emotion. The experiments in this thesis focus on the structural and functional hippocampal changes in the 1AKO mouse compared to wild-type mice in order to elucidate the cellular mechanisms behind the alterations in behavior. Electrophysiology was used in the CA1 region of the hippocampus to show that pyramidal neurons in the 1AKO mouse receive less glutamatergic input than control mice during the critical period, resulting in decreased AMPA-mediated excitation and LTP in the adult. Interestingly, morphological analyses demonstrated a significant enhancement in proximal dendritic branching in both the juvenile and adult 1AKO mouse that may be the result of the developmental effects of increased serotonergic efflux. Additional experiments focused on the role of corticotropin-releasing factor (CRF) in the 1AKO mouse, based on the fact that peak hippocampal levels of this neuropeptide coincide in time with the critical period of development when 1AR deletion has pronounced effects. We found that adult 1AKO mice showed increased numbers of CRF-containing interneurons and that CRF1 receptor antagonism restored CA1 LTP to control levels. Taken together, these results reveal a complex interplay of decreased synaptogenesis and number of AMPA receptors, and excessive activation of CRF1 receptors that may underlie the cognitive deficits and anxiety-like behavior of the 1AKO mouse. The experiments support the continued research into the neurobiological mechanisms of human anxiety disorders.

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HIPPOCAMPAL CIRCUITRY DYSFUNCTION IN THE

5-HT1A KNOCKOUT MOUSE

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ABSTRACT

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Kayla Lynn Metzger
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Anxiety disorders are the most prevalent class of mental illness, yet currently available treatments are often ineffective or inadequate, leaving many patients with lingering symptoms. The serotonin 1A receptor (1AR) has been implicated in the etiology of these disorders, which often show comorbidity with cognitive dysfunction. Mice with the 1AR genetically deleted or “knocked out” (1AKO) during a critical period in development (postnatal days 13-21) exhibit anxiety-like behavior and learning and memory deficits, and may therefore represent a useful genetic model in studying the neurobiological effects of this receptor. The hippocampus has been shown to highly express the 1AR and to be a key mediator in memory and the regulation of emotion. The experiments in this thesis focus on the structural and functional hippocampal changes in the 1AKO mouse compared to wild-type mice in order to elucidate the cellular mechanisms behind the alterations in behavior. Electrophysiology was used in the CA1 region of the hippocampus to show that pyramidal neurons in the 1AKO mouse receive less glutamatergic input than control mice during the critical period, resulting in decreased AMPA-mediated excitation and LTP in the adult. Interestingly, morphological analyses
demonstrated a significant enhancement in proximal dendritic branching in both the juvenile and adult 1AKO mouse that may be the result of the developmental effects of increased serotonergic efflux. Additional experiments focused on the role of corticotropin-releasing factor (CRF) in the 1AKO mouse, based on the fact that peak hippocampal levels of this neuropeptide coincide in time with the critical period of development when 1AR deletion has pronounced effects. We found that adult 1AKO mice showed increased numbers of CRF-containing interneurons and that CRF₁ receptor antagonism restored CA1 LTP to control levels. Taken together, these results reveal a complex interplay of decreased synaptogenesis and number of AMPA receptors, and excessive activation of CRF₁ receptors that may underlie the cognitive deficits and anxiety-like behavior of the 1AKO mouse. The experiments support the continued research into the neurobiological mechanisms of human anxiety disorders.
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CHAPTER 1:

GENERAL INTRODUCTION
ANXIETY DISORDERS

Anxiety is a normal, adaptive reaction to stressful situations. However, when anxiety becomes excessive or irrational it can become a chronic, disabling problem. The five major types of anxiety disorders are Generalized Anxiety Disorder (GAD), Post-traumatic Stress Disorder (PTSD), Obsessive-Compulsive Disorder (OCD), Panic Disorder, and Social Phobia. Collectively, these disorders are the most common class of mental illness in the general population (Kessler et al, 2009). Anxiety disorders show a lifetime prevalence of 28.8% of the US adult population, with a mean onset at age 11 years (Kessler et al, 2005a). The estimated societal cost of anxiety disorders in the US was approximately $42.3 billion in the early 1990s, representing an enormous burden at nearly one-third of the total $148 billion mental health bill (Greenberg et al, 1999). These disorders are also strongly associated with suicidal ideation and suicide attempts. One large-scale study showed that among those who had attempted suicide, 64.1% had at least one anxiety disorder (Sareen et al 2005).

Anxiety disorders have a high incidence of comorbid psychiatric and physical illnesses. Approximately half of patients with GAD also have Major Depressive Disorder (MDD) and vice versa (Grant et al, 2005; Kessler et al, 2005b). Furthermore, both anxiety and depression are associated with cognitive deficits, particularly learning and memory dysfunctions (Bremner et al, 1993; Deckersbach et al, 2011; Deckersbach et al, 2000; Hickie et al, 2005; Johnsen and Asbjornsen, 2008; Mantella et al, 2007; Naismith et al, 2006).
In spite of a number of efficacious treatments available to those who suffer from these disorders, it is estimated that as many as 40% of patients find no relief from current treatments, and many more have lingering symptoms (Bandelow et al, 2004; Cowley et al, 1997; Pallanti et al, 2002). Of the two classes of drugs most commonly prescribed for anxiety, benzodiazepines can induce sedation, memory loss, tolerance, and dependence, while selective serotonin reuptake inhibitors (SSRIs) are characterized by high levels of relapse and adverse side effects, and a delayed therapeutic onset of several weeks (Cryan and Sweeney, 2011; Ravindran and Stein, 2010; Sartori et al, 2011). This highlights the need for researchers to better understand the etiology of anxiety in order to identify new targets for therapeutic intervention.

**Animal models of anxiety**

Given the ethical and legal issues involved in human brain research, most preclinical work on psychiatric disease focuses on animal studies. Psychiatric disorders are likely caused by a complicated interplay of genetic and environmental factors. In view of the complexity and our poor understanding of the biological basis of these disorders, it would be impossible to model the full human psychiatric condition in animals. Scientists take advantage of genes and structures that are conserved across species to study simplified conditions in a controlled environment in order to further our knowledge of the causes and treatment possibilities for psychiatric disorders. Animal models used by researchers may share the etiology, symptoms, or treatment
responsiveness of human disease, although any single model is unlikely to replicate all aspects of the disease process.

The laboratory mouse is a powerful system for biomedical research, and is the most widely used mammalian model. In spite of outward appearances, mice are remarkably similar to humans in physiology, anatomy, and genetics. Approximately 99% of mouse genes have a detectible human homolog, and vice versa, making mice ideal for use in genetic studies (Sands, 2003). A short lifespan and rapid reproductive rates facilitate the ability for researchers to study a large number of individuals across many lifetimes, and generated gene mutations can be maintained through selective breeding using large numbers of mice at relatively little expense. Furthermore, inbred strains minimize subject variation and provide controls for finely-tuned genetic manipulations.

Tests of behavioral anxiety in animals

Human anxiety cannot be fully duplicated in the mouse because we simply do not know the true emotions of non-human animals, or whether they feel anxious or depressed. However, aside from the human cerebral cortex, vertebrate brains have structural and organizational similarities which allow comparisons to be made across species (Cryan and Holmes, 2005; Jones, 2002; Tecott, 2003). Particularly among mammals, neural structures and the circuitry that connects them have been evolutionarily conserved. Therefore, in addition to genetic similarities with humans, mice have comparable behavioral and physiological responses to fearful stimuli.
A number of tasks have been developed for mouse research, which provoke these behavioral or physiological responses in order to study the neural circuits and genetic factors underlying human disease states. Many current rodent tests of anxiety-related behavior fall into two major categories. Approach-avoidance tasks are based on a balance between the innate rodent desire to explore, and their fear of open, elevated, or illuminated areas. These tasks include the open field test, the elevated plus maze, and the light/dark box. Mice in these tasks that are considered anxious tend to stay away from aversive areas to a greater degree than the appropriate control. These tests have face validity in that human anxiety disorders are often characterized by avoidance of feared or threatening stimuli or situations. The power behind these experiments comes from their predictive validity, in that avoidance behavior is reduced by treatment with clinically effective anxiolytics, and intensified by anxiogenic drugs that cause anxious feelings in humans (Belzung and Griebel, 2001; Lister, 1990).

Another commonly used type of behavioral task involves Pavlovian conditioned fear responses, which are used to model the cognitive aspects of depression and anxiety disorders (Fendt and Fanselow, 1999; Maren, 2001). These tasks involve repeated pairings of an innocuous stimulus or environment with a threatening or painful stimulus so that the rodent eventually displays fearful behavior when presented with the former. Although mainly a learning and memory task, conditioned fear is known to activate the same neural circuitry involved in human anxiety and depression, thereby providing construct validity to this line of testing (LeDoux, 2000). Therefore these tasks may be used to model both cognitive and emotional aspects of human disorders. Conditioned
fear responses, particularly the extinction of the fear response, are sensitive to drugs that also work clinically to extinguish panic or phobias. (Davis, 1990; Ledgerwood et al, 2005; Ressler et al, 2004).

Anxiety is a highly adaptive and conserved response to a potential threat across species, and anxiety disorders can be considered to be the pathological end of the spectrum of this normal, adaptive behavior. A person with an anxiety disorder exhibits fear or panic in situations where most people would not feel anxious or threatened. On the other hand, depression is a clinically defined human disease state with psychological symptoms such as feelings of hopelessness, despair, or anger, and often accompanied by low energy and other neuroendocrine and somatic symptoms. Therefore what is assayed in the mouse are specific, measurable behaviors known as endophenotypes that appear relevant to human depression (Holmes, 2003). The most commonly used behavioral tasks for depression are the forced swim test and tail suspension test, both of which use immobility as the dependent variable. These tests have both face validity and some amount of predictive validity: they are sensitive to clinically efficacious antidepressants, however these drugs reduce immobility after acute exposure, whereas only chronic exposure is effective clinically (Duman, 2010; El Yacoubi et al, 2003; Naudon et al, 2002). The novelty suppressed feeding paradigm measures a rodent’s aversion to eating in a novel environment. Although lacking in face validity, this test has high predictive validity in that it is sensitive to acute anxiolytics and chronic antidepressants, but not acute antidepressants (Bodnoff et al, 1988; Bodnoff et al, 1989; Guilloux et al, 2011).
Although many of these tests have been standardized and validated to some extent, there are limitations to every model. Several studies have shown that results of behavioral tasks can be sensitive to even subtle variations in testing procedure, housing conditions, and laboratory environment (Crabbe et al, 1999; Cryan and Mombereau, 2004; Wahlsten et al, 2003). Furthermore, as newer classes of drugs are made available, researchers may need to utilize novel behavioral tasks to test them.

Animal models of anxiety: environmental and genetic

An animal model can be defined as an (non-human) organism or particular state of an organism that reproduces aspects of the human pathology, providing some degree of predictive validity. On the other hand, a test, such as those described above, provides only an end-point behavioral or physiological measure designed to assess the effect of a genetic, pharmacological, or environmental manipulation (Urani et al, 2005). Several rodent models have been designed to study the contribution of environmental factors towards the development of anxiety disorders. Most types of anxiety disorders are thought to involve exposure to prolonged or recurring stressors rather than to a single acute event. By repeatedly activating the stress pathways, these models reproduce conditions under which pathological anxiety might develop. Many models use stressors designed to provoke fear behaviors. For example, chronic stress paradigms such as exposure to predator odors or an aggressive conspecific elicits defensive activities or postures (Blanchard et al, 2005; Blanchard et al, 2003; Slattery et al, 2012; Staples, 2010). Pharmacological activation of stress pathways can also induce long-term
behavioral changes in tests of anxiety (Lee et al, 2008). Early-life stress such as maternal
deprivation is also known to produce anxiety-like behavior that manifests in the juvenile
or adult animal (Rice et al, 2008; Sherrin et al, 2009; Troakes and Ingram, 2009). This
model suggests developmental experiences may result in life-long behavioral changes.
Since the median age of onset in human anxiety disorders is 11 years, there is great
interest in understanding the developmental aspects of the disorder and focusing on early
interventions (Kessler et al, 2005a).

The genetics of anxiety disorders are recognized to be complex, polygenic, and
epistatic, which have made them challenging to model in a straightforward way (Lesch,
2001). One traditional method has been to selectively breed animals for high versus low
trait anxiety behavior, allowing for the identification of candidate genes correlated with
the human disorder (Landgraf and Wigger, 2002; Muigg et al, 2009; Wegener et al,
2012). Environmental stressors may further differentiate these extremes, suggesting that
animals bred for high anxiety behavior may be more vulnerable to the effects of stress
(Savignac et al, 2011; Stedenfeld et al, 2011).

The targeted deletion or “knockout” of a single gene is currently used as one of
the primary approaches to elucidate the genetic basis of anxiety disorders. The classic
constitutive knockout has the gene mutation present in all cells and throughout the
lifetime of the animal, including during development. Some gene knockouts are
developmentally lethal, with deletion of an essential gene resulting in death of the animal
during the embryonic or early postnatal stage. When the mouse survives to adulthood,
there may be multiple effects from the mutation, which can complicate the interpretation of neurobiological research. Compensatory mechanisms in other neural systems may obscure the function of the gene. However, the developmental effects following gene deletion can themselves provide important insights into genetic function as well as into the plasticity of neural systems. Since human anxiety disorders often present with an early age of onset (average age 11 years), this disease class is particularly suited for this strategy (Kessler et al, 2005a). The constitutive knockout mouse is a powerful method of modeling the genetic contribution to a complex neurological disorder.

The 5-HT1A receptor

Brain serotonin (5-HT) plays a major role in a number of physiological processes including the regulation of mood, impulse control, sleep, eating, libido, and cognitive functions such as learning and memory. 5-HT is synthesized from the amino acid L-tryptophan in serotonergic neurons in the raphe nuclei and released widely throughout the cortex and limbic systems. There are 7 distinct families and at least 14 known 5-HT receptor subtypes, many of which can be further divided into subpopulations (Berger et al, 2009; Nichols and Nichols, 2008).

The 5-HT1A receptor (1AR) is the most extensively distributed of the 5-HT receptors, showing high density in raphe nuclei, hippocampus, amygdala, and septum (el Mestikawy et al, 1991). Low to moderate levels of the 1AR are also seen in cerebral cortex, basal ganglia, hypothalamus, and thalamus (Kusserow et al, 2004). All except one family of 5-HT receptors are G-protein coupled receptors, with the 1AR associated
with G\textsubscript{i}/G\textsubscript{o}. Activation of the 1AR causes the opening of G-protein-gated inwardly-rectifying potassium (GIRK) ion channels, hyperpolarizing the cell and making it less likely to fire an action potential. Additionally, activation of this G-protein inhibits adenylate cyclase, which decreases levels of the second messenger cAMP and results in decreased transcription.

The 1AR functions as both a presynaptic autoreceptor and a postsynaptic heteroreceptor (Blier et al, 1987). Presynaptic autoreceptors are located on the cell body and dendrites of serotonergic neurons in the raphe nuclei where they serve to negatively inhibit their own activity and suppress the release of serotonin (Aghajanian and Lakoski, 1984). Postsynaptic heteroreceptors are found mainly on glutamatergic and GABAergic pyramidal cells in limbic and cortical regions innervated by serotonergic raphe cells, where they inhibit the firing of target neurons and regulate the function of other neurotransmitter systems (Azmitia et al, 1996; Hall et al, 1997; Palchaudhuri and Flugge, 2005).

**The 5-HT1A receptor in anxiety**

The first evidence for the role of the 1AR in anxiety came in 1979 with a clinical study showing the anxiolytic properties of buspirone, a 1AR partial agonist (Goldberg and Finnerty, 1979). Since then, SSRIs have been shown to be effective in many cases in the treatment of anxiety disorders (Ballenger, 1999; Liebowitz, 1999). This class of drugs blocks the 5-HT transporter and is thought to work by increasing the synaptic level of serotonin (Nutt et al, 1999). There is clinical evidence that 5-HT1A antagonists or
partial agonists can improve the effectiveness of SSRIs by halting the 1A autoreceptor inhibition of cell firing that SSRIs induce early in treatment (Artigas et al, 2006; Stahl, 1997).

In addition to treatment studies, clinical findings from genetic analyses and imaging data have shown the 1AR to be involved in anxiety disorders. For example, patients with panic disorder show decreased 1AR binding in PET imaging studies (Neumeister et al, 2004). A single nucleotide polymorphism (SNP) that occurs in the transcriptional control region of the human 1AR gene is associated with anxiety-related personality traits, severe depression, and suicide (Lemonde et al, 2003; Strobel et al, 2003). This allele abolishes repression of 1AR expression to reduce serotonergic neurotransmission.

Recent evidence also shows a role for the 1AR in cognitive deficits and mood associated with dementia. PET imaging has shown significantly decreased 1AR density in the hippocampi of patients with mild cognitive impairment (24% average loss) or Alzheimer’s disease (49% average loss) (Kepe et al, 2006).

*5-HT1A receptor knockout mouse*

In 1998, three lines of 1AR knockout mice were independently created on different genetic backgrounds and tested under similar conditions in a variety of anxiety-like behavioral tasks (Heisler et al, 1998; Parks et al, 1998; Ramboz et al, 1998). Each of these were found to have increased levels of fear, avoidance, and stress responses, along
with decreased exploratory behavior and antidepressant-like effects. Subsequent findings also demonstrated impairments in hippocampal-dependent learning and memory tasks such as the Morris water maze and contextual fear conditioning (Klemenhagen et al., 2006c; Sarnyai et al., 2000). However, in learning tasks mediated by the prefrontal cortex, such as the serial reversal learning paradigm, 1AKO mice performed similarly to wild-type (WT) mice (Pattij et al., 2003; Wolff et al., 2004).

In many behavioral anxiety tests, the 1AKO mouse responds to treatment with therapeutics used to treat anxiety and depression in humans, showing that this model has predictive validity for human affective disorders. One background strain of the 1AKO mouse was discovered to be insensitive to the anxiolytic effects of benzodiazepines, which was attributed to a pathway from 1AR deficit to GABA_A receptor subunit abnormalities (Sibille et al., 2000a). This strain may be useful for modeling benzodiazepine treatment-resistant anxiety. However, the anxiolytic effects of 8-OH-DPAT and SSRIs are blocked in the 1AKO in the novelty-suppressed feeding task, which is sensitive to acute anxiolytics and chronic but not acute antidepressants. This indicates that SSRIs exert their anxiolytic effects by activating 1ARs (Santarelli et al., 2003).

Prior studies using the 1AKO mouse have shown there to be a critical period for the development of anxiety-like behavior. Genetic manipulations of 1AR expression demonstrated that deletion of this receptor in the forebrain of the adult mouse did not produce an anxiety phenotype (Gross et al., 2002). This is consistent with the inability of 1AR antagonist WAY-100635 to produce anxiety-like behavior in the adult mouse (Cao
and Rodgers, 1997; Fletcher et al, 1996). However, when mice had 1AR expression disabled in forebrain regions during the embryonic and postnatal period (E0-P21), they were behaviorally indistinguishable from 1AKO mice as adults (Gross et al, 2002). Furthermore, pharmacological blockade from P13-P34 resulted in anxiety-like behavior in the adult mouse, thereby narrowing the critical time for the development of anxiety-like behavior in the 1AKO to P13-P21 (Lo Iacono and Gross, 2008).

A mouse line was recently developed for conditional deletion of the 1AR both spatially (autoreceptor versus heteroreceptor) and temporally (whole-life versus adult). Deletion of the autoreceptor throughout life produced a mouse that had an anxious phenotype. In contrast, lifetime elimination of the heteroreceptor led to a non-anxious mouse that exhibited a depression-like phenotype (Richardson-Jones et al, 2011). When the autoreceptor was knocked out only during adulthood, mice exhibited depression-like behavior, but adult suppression of the heteroreceptor resulted in no behavioral abnormality (Richardson-Jones et al, 2010; Richardson-Jones et al, 2011). The autoreceptor is therefore important during development for the predisposition to anxiety whereas the heteroreceptor is important for the expression of behavioral depression. However, changes that occur in forebrain regions following deletion of the autoreceptor may contribute towards the anxiety-like behavior and cognitive deficits seen in the 1AKO.

In mice with 1AR constitutive genetic deletions, compensatory effects in serotonin, other serotonin receptors, or other neurotransmitter systems may alter neural
circuitry and induce the behavioral abnormalities seen in these mice. For example, previous studies have shown that 5-HT1B receptors in the striatum appear to increase their responsiveness in response to 1AR deletion (Knobelman et al., 2001a). In fact, some of the most interesting or important findings that may be gained from constitutive deletions are the compensatory or related consequences of life-long changes in the target gene.

Goals of thesis research

The primary goal of this thesis was to characterize hippocampal dysfunction on a cellular level that might contribute to the behavioral anxiety and cognitive deficits in the 1AKO mouse. These studies were undertaken because of the pivotal yet poorly understood role the 1AR plays in the development and treatment of anxiety disorders. The 1AKO mouse shows anxiety-like behavior along with cognitive deficits similar to those that are often comorbid in patients with anxiety disorders. Prior studies in the 1AKO mouse have demonstrated a period in development that is crucial to formation of the behavioral phenotype, indicating utility as a model for genetic predisposition to anxiety. I used cellular and electrophysiological techniques to investigate alterations in hippocampal cellular characteristics and activity that result from genetic deletion of the 5-HT1A receptor.

Clinical and animal studies have shown the hippocampus to be a key region in the regulation of mood. This area of the brain is also known to be crucial to various aspects of learning and memory. By studying alterations in the hippocampal circuitry of the
1AKO mouse, I hoped to determine the cellular mechanism by which deletion of the 1AR affects behavior and cognitive abilities. Chapter 2 describes my evaluation of cellular properties and synaptic activity of CA1 pyramidal cells using whole-cell patch-clamp and extracellular electrophysiology, and single-cell morphological analyses. Previous studies show increased hippocampal theta activity in anxiety-provoking tasks in the 1AKO mouse, and that 1AR agonists and anxiolytics decrease hippocampal activity (Adhikari et al, 2011; Gordon et al, 2005b; Hirose et al, 1990; Tada et al, 1999; Zhu and McNaughton, 1994). However, the learning and memory deficits of the 1AKO indicate that hippocampal activity may be reduced. Based on reports that 1AKO hippocampal activity is state-dependent, I examined both the baseline and stimulated hippocampal systems to resolve this discrepancy.

The experiments described in the Appendix examine the effects of CRF or a CRF₁ receptor antagonist on the same cellular characteristics and activity, as well as the expression of CRF in the 1AKO mouse hippocampus. The CRF neuropeptide and CRF₁ receptor have been shown to peak in expression in the rodent hippocampus during the same critical time period when the 1AR is necessary for normal behavior in the adult mouse. Therefore, I hypothesized that CRF plays an important role in the development and maintenance of the behavioral phenotype of the 1AKO mouse.

These studies were designed to advance the knowledge of the development of anxiety disorders. With continued research, these findings could help uncover more effective treatments for human anxiety disorders and cognitive dysfunction.
CHAPTER 2:

ALTERED SYNAPTOGENESIS DURING DEVELOPMENT MAY UNDERLIE HIPPOCAMPAL CIRCUITRY CHANGES AND IMPAIRED MEMORY IN THE 5-HT1A KNOCKOUT MOUSE

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ABSTRACT

The serotonin 1A receptor is linked to the etiology of mood disorders and cognitive deficits. Mice in which the 5-HT1A receptor has been genetically deleted (1AKO) during a limited developmental period demonstrate a robust anxiety phenotype and deficits in learning and memory. The CA1 region of the hippocampus shows the greatest expression of 5-HT1A receptors, and has been shown to be crucial to development of the behavioral phenotype. This study used electrophysiology and morphological analyses to examine differences in cell properties and synaptic activity in the juvenile and adult 1AKO mouse and their wild-type (WT) controls. We showed that both juvenile and adult 1AKO mice displayed increased dendritic branching in CA1 pyramidal cells compared to WT mice. Adult 1AKO mice showed cellular characteristics that reflect this change in morphology, as well as deficits in LTP and reduced AMPA receptor-mediated responses compared to the WT mice. In contrast, juvenile mice exhibited no differences in cell membrane properties but showed decreased glutamatergic input. Together these data indicated that morphological differences in the 1AKO already in place at the beginning of the critical period drive alterations in synaptic development that led to deficits in the adult. These changes may underlie the behavioral phenotype of the 1AKO mouse.
INTRODUCTION

The serotonin 1A receptor (1AR) has been linked to the etiology and treatment of mood disorders and cognitive deficits. Clinical studies have demonstrated a 1AR deficiency in people with anxiety or depression (Drevets et al, 1999; Lesch et al, 1992; Meltzer and Maes, 1995; Sargent et al, 2000; Savitz et al, 2009). Patients with panic disorder, a form of anxiety, and those with Alzheimer’s disease, show decreased 1AR binding in PET imaging studies (Kepe et al, 2006; Neumeister et al, 2004; Truchot et al, 2008). An allelic variation in the 1AR gene that abolished repression of a transcriptional regulator is associated with anxiety-related personality traits (Strobel et al, 2003). Similarly, animals studies have shown that constitutive genetic deletion, or knockout, of the 1AR in mice (1AKO) results in an anxiety-like phenotype, and learning and memory deficits in the adult animal (Heisler et al, 1998; Klemenhagen et al, 2006b; Parks et al, 1998; Ramboz et al, 1998; Sarnyai et al, 2000).

1ARs are inhibitory G-protein coupled receptors found as autoreceptors on serotonergic cells in the raphe nuclei, and as heteroreceptors on non-serotonergic neurons in raphe and forebrain regions. The 1AR exhibits highest expression in the CA1 region of the hippocampus (Chalmers and Watson, 1991; Gross et al, 2002). Gross et al (2002) demonstrated that forebrain-specific ectopic expression of 1AR is sufficient to rescue the anxiety-like phenotype of 1AKO mice. In these forebrain 1AKO “rescue” mice, the pattern of the rescued receptor was similar to wild-type (WT) mice only in the hippocampus. Recently, a mouse line was developed for selective elimination of the 1AR in spatial (autoreceptor versus heteroreceptor) and temporal (whole-life versus adult)
roles. The knockout of the autoreceptor throughout life produced a mouse that had an anxious phenotype but not reduced behavioral despair. In contrast, lifetime elimination of the heteroreceptor led to a non-anxious mouse that exhibited a depression-like phenotype (Richardson-Jones et al., 2011). When the autoreceptor was knocked out only during adulthood, mice exhibited depression-like behavior, but adult suppression of the heteroreceptor resulted in no behavioral abnormality (Richardson-Jones et al., 2010; Richardson-Jones et al., 2011). The autoreceptor is therefore important during development for the predisposition to anxiety whereas the heteroreceptor is important for the expression of behavioral depression.

Changes driven by the heteroreceptor that occur in the hippocampus during development are important for behavioral despair. Other changes may be important for predisposition to anxiety or depression, possibly due to altered 5-HT input in the autoreceptor knockout. For example, serotonin efflux is increased in some brain regions, hippocampal pyramidal neurons demonstrate increased proximal dendritic branching, theta power is augmented during an anxiety test, and GABA receptor subunits are altered (Ferreira et al., 2010b; Gordon et al., 2005a; Parsons et al., 2001; Richardson-Jones et al., 2011; Sibille et al., 2000b). In addition, 1AKO mice have deficits in hippocampal-mediated learning and memory tasks, but not in memory tests that are mediated by other brain regions (Klemenhagen et al., 2006a; Sarnyai et al., 2000). These experiments demonstrate that the hippocampus is crucial to the formation of the phenotype in the adult 1AKO animal. However, the cellular mechanisms underlying those changes are unknown.
The critical period for development of anxiety-like behavior in the 1AKO occurs within a defined temporal window. Genetic manipulations showed that turning off 1AR expression in the forebrain of the adult mouse did not produce an anxiety-like phenotype (Gross et al, 2002). This is consistent with the inability of 1AR antagonist WAY-100635 to produce anxiety-like behavior in the adult mouse (Cao et al, 1997; Fletcher et al, 1996). However, mice with 1AR expression disabled in forebrain regions during the embryonic and postnatal period (E0-P21) are behaviorally indistinguishable from 1AKO mice as adults (Gross et al, 2002). A report using pharmacological blockade with WAY-100635 showed that 1AR activity is necessary from P13-P34 for normal adult behavior, thereby narrowing the critical time for the development of anxiety-like behavior in the 1AKO to P13-P21 (Lo Iacono et al, 2008).

The present study used whole-cell patch-clamp and extracellular electrophysiology from hippocampal slices as well as morphological analyses to evaluate the differences in cellular characteristics, excitatory, and inhibitory activity between 1AKO and WT mice at the beginning of the critical period and in adulthood. Our findings suggest that changes taking place in the hippocampus of the 1AKO mouse during this critical period contribute to cognitive impairments in the adult.

MATERIALS AND METHODS

Animals
Founders from an established colony in the laboratory of Dr. Mark Geyer, University of California, San Diego, CA were obtained to generate a colony at Children’s Hospital of Philadelphia. Male offspring from heterozygous pairings were used in this study. Litters were weaned, separated by sex, and genotyped at 3 weeks of age. For all experiments, adult (2-5 months) and juvenile (12-14 postnatal days) homozygous wild-type (WT) and knockout (1AKO) male mice on a 129/Sv background (Ramboz et al, 1998) were used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional IACUC committee.

Electrophysiology

Brain slices were prepared as previously described (Richardson-Jones et al, 2011; Tsetsenis et al, 2007). Briefly, mice were decapitated and their brains dissected out in cold artificial cerebrospinal fluid (aCSF) in which NaCl was replaced with sucrose (248mM). The forebrain was blocked and cut on a Leica VT1000s vibratome (Leica Microsystems, Bannockburn, IL). Coronal slices (200 μm thick for whole-cell patch-clamp, 400 μm for field recordings) containing hippocampus were placed in aCSF (in mM, 124 NaCl, 3.2 KCl, 1.25 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 dextrose and 26 NaHCO3) at 37 °C bubbled with 95% O2/5% CO2. After one hour, slices were kept at room temperature. Individual slices were placed in a recording chamber and perfused with aCSF at 2 ml/min at 32 °C maintained by an in-line solution heater (TC-324, Warner Instruments). Neurons were visualized using a Nikon E600 upright microscope,
and signals were collected and stored using a Multiclamp 700B, Digidata 1322A analog-to-digital converter, and pClamp 9.0 software (Molecular Devices, Sunnyvale, CA). All drugs were made in stock solutions, diluted on the day of the experiment, and added directly to the aCSF.

Whole-cell recordings were obtained using electrodes filled with an intracellular solution of (in mM) 130 K-gluconate, 5 NaCl, 10 Na phosphocreatine, 1 MgCl₂, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.5 Na₂GTP, biocytin 0.1%, pH 7.3 for measuring EPSCs, and 70 K-gluconate, 70 KCl, 2 NaCl, 10 Na phosphocreatine, 4 EGTA, 10 HEPES, 4 MgATP, 0.3 Na₂GTP, biocytin 0.1%, pH 7.3 for measuring IPSCs. For evoked PSCs, a cut was made between CA3 and CA1 to prevent recurrent excitation. CA1 pyramidal neurons were patched and cell characteristics recorded using current clamp techniques as previously described (Beck et al, 2004). Voltage clamp recordings were conducted holding the membrane potential at -60 mV (Lemos et al, 2011). To isolate AMPA-mediated eEPSCs, bicuculline methiodide (BMI, 20 µM) and (2R)-amino-5-phosphonopentanoate (AP5, 30µM) were added to the superfusing aCSF. GABA-A-mediated IPSCs were isolated similarly with the addition of AP5 and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20µM).

For extracellular evoked potential recording, glass electrodes were filled with 4M NaCl and placed in stratum radiatum of area CA1. The Schaffer collateral afferent pathway was stimulated with a concentric bipolar platinum/iridium electrode (FHC, Bowdoin, ME) and field excitatory postsynaptic potentials (fEPSPs) were recorded. Input/output curves from 0.001 to 1 mA were generated and the stimulation that produced
half maximal response was used. Baseline potentials were monitored for 30 min before
tetanic stimulation. Long-term potentiation (LTP) was induced by four trains of
conditioning stimulation (1 s at 100 Hz) at 30-s intervals (high-frequency stimulation
(HFS)). Responses were subsequently followed for 60 min at 20-s intervals.

Data analysis

MiniAnalysis software (Synaptosoft, Inc., Decatur, GA) was used to analyze spontaneous
and miniature EPSC events for frequency, amplitude, rise time, decay time, baseline
holding current, and area. The slope and amplitude of fEPSP and ePSCs were measured
using pClamp 9 software. fEPSP was expressed as percent of pre-tetanus baseline
values, and ePSCs expressed as percent of maximal stimulus before an action potential
was elicited. LTP was quantified as the mean of the last 10 min of 60 min post-tetanus.
Statistica software was used for all statistical tests. Passive and active membrane
characteristics were analyzed using Clampfit software (Molecular Devices) and then
compared between WT and 1AKO using unpaired Student’s t-test. Repeated measures
ANOVA was used to examine frequency-intensity (F-I) plot and all evoked responses,
followed by Tukey post-hoc analysis.

Neuron morphology
After electrophysiological recording, slices were placed in 4% paraformaldehyde and stored at 4°C until staining. To visualize the biocytin-filled neuron, slices were incubated in streptavidin-conjugated Alexa Fluor 647 (1:200; Invitrogen) in PBS with 0.25% Triton X-100 and 0.5% bovine serum albumin for 90 min at room temperature. After several washes, sections were mounted on Superfrost slides and coverslipped with Fluoromount-G mounting media (Southern Biotech, Birmingham, AL). Labeled cells were visualized using a Leica confocal DMIRE2 microscope (Leica, Allendale, NJ). A 20X scan consisting of several serial, optical sections (0.6 μm) was acquired at the level of the cell body of the biocytin-labeled neuron. For morphological analysis, the total number of optical sections taken during the scan depended on the length of the dendrites and the depth that the dendrites traveled through the section, as more optical sections were needed to scan longer, deeper extending dendrites. The entire extent of the dendritic tree of each neuron was obtained for analysis. Images were captured using a digital camera and Leica Confocal software (Version 2.5, Leica). The xyz confocal stacks were collected and analyzed using the Neurolucida software program (MicroBrightfield, Williston, VT).

RESULTS

Cellular properties in the adult mouse

Cellular characteristics were measured in 26 WT and 28 1AKO pyramidal cells from a total of 11 WT and 13 1A KO mice in the CA1 region of the hippocampus. Table
Input resistance (p<0.001) and tau (p<0.05) were significantly higher in the 1AKO as compared to the WT, showing possible differences in morphological characteristics. There was no difference between the genotypes in resting membrane potential (RMP) or action potential (AP) threshold. However, the activation gap (i.e. the difference between the resting membrane potential and the action potential threshold) was significantly smaller in the 1AKO, indicating a higher intrinsic excitability in these cells. AP duration was longer in the 1AKO compared to WT cells (p<0.005). A frequency-intensity plot was constructed from the mean firing frequency in response to square current pulses (400 ms in duration) of increasing amplitude (20 pA steps starting at 0 pA up to 100 pA). 1AKO neurons demonstrated increased excitability compared with those of WT cells, in that they reached higher firing rates given the same magnitude of input (Figure 1B). This difference was significant at current steps ranging from 70 to 100 pA (p<0.05).

Spontaneous excitatory post-synaptic currents (sEPSCs) were measured in voltage clamp at -60mV. The results are summarized in Table 2 (means ± SEM). The frequency of excitatory events did not differ between 1AKO and WT pyramidal cells, however sEPSC amplitude was greater in the WT cells as compared to the 1AKO cells (p<0.001). Both rise and decay times of sEPSCs were longer in the 1AKO neurons than in the WT cells (p<0.005 and p<0.05 respectively). In spite of these differences in kinetics, the average charge per event, as defined by the area under the curve, was significantly smaller in the 1AKO cells (p=0.005). When this value was multiplied by the sEPSC frequency to get the mean phasic current, it was also significantly reduced in the 1AKO.
neurons as compared to the WT cells (p<0.05), indicating that 1AKO cells receive less total glutamatergic input.

In these recordings of sEPSC activity, larger amplitudes were correlated with faster rise times only in the WT pyramidal neurons ($R^2=0.47$, p<0.001). This indicated that these cells exhibited electrotonic filtering (Ling & Benardo, 1999, Crawford et al 2011). To determine if differences in sEPSC characteristics between WT and 1AKO cells were due to the activity of local neurons within the slice, recordings were done in a small subset of cells in the presence of the AP blocker tetrodotoxin (1µM), and represent the spontaneous release of neurotransmitter at the synapse. Miniature EPSCs (mEPSCs) in the WT cells (N=6) displayed significantly lower amplitude (p<0.01) and phasic current (p<0.05) as compared to sEPSCs, as well as increased mEPSC decay time (p<0.05). The decreased amplitude indicated that AP-independent component makes up a small portion of the total glutamatergic input to these cells. Conversely, there were no differences between mEPSCs and sEPSCs in the 1AKO (N=5) cells, showing that AP-independent input occurs in greater proportion in 1AKO neurons. However, the AP-independent glutamatergic input displayed electrotonic filtering in both the WT ($R^2=0.70$, p<0.05) and 1AKO ($R^2=0.96$, p<0.005) cells.

In a separate set of experiments, spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded in the presence of DNQX and AP5 in order to isolate GABAergic input (Table 2). The rise time (p<0.05) and slow decay time (p<0.05) were significantly longer in 1AKO cells as compared to WT cells. This difference in kinetics indicates potential alterations in GABA$\text{A}$ receptor subunits between the genotypes.
sIPSC frequency, amplitude, fast decay time, average charge per event, and phasic current did not differ significantly between 1AKO and WT cells. Overall, 1AKO pyramidal cells exhibited greater intrinsic excitability but decreased glutamatergic input as compared to WT neurons, and evidence for changes in AMPA and GABA_A receptor subunits.

**Cellular properties in the juvenile mouse**

Whole-cell electrophysiology was performed on 20 WT and 14 1AKO cells from 5 WT and 5 1AKO P13 mice. Unlike the adult pyramidal cells, there were no significant differences in passive or active membrane properties between the P13 WT and 1AKO cells (Table 1). Additionally, there were no differences in excitability of pyramidal cells between the genotypes as evidenced by the F-I plot (Figure 1A). This indicates that ion channels in the P13 mouse are still developing. Further evidence for this comes from a significantly higher resistance in the P13 pyramidal cells as compared to that in adult (p<0.0001 for both genotypes). Analysis of sEPSCs showed that 1AKO cells have a lower frequency of excitatory events than WT neurons (p<0.05), signifying that 1AKO neurons receive less glutamatergic input at this time (Table 2).

**Morphology in the adult mouse**
Morphological analysis was conducted on 19 WT and 24 1AKO pyramidal cells that were filled with biocytin during whole-cell electrophysiology recordings (Figure 2). 1AKO neurons had a greater number of dendritic branches than WT cells (p<0.0001, Figure 2C), as well as a higher number of nodes (p<0.0001) and ends (p<0.0001). The total dendritic length when all processes were summed also differed between genotypes, with 1AKO cells having significantly longer total dendrites than WT cells (p<0.0001, Figure 2D).

A Sholl analysis was performed to determine the number of times the dendrites crossed each radial segment (20µ) as well as the length of each dendritic segment within each radii (Figure 2E). The dendritic trees of 1AKO neurons were significantly more complex than those of WT cells. The number of intersections within each radii was higher in the 1AKO cells (p<0.0001, Figure 2F), as was dendritic length within each segment (p<0.0001). Post-hoc analyses showed that this significant increase in complexity occurred from 60 to 180µ from the soma. This indicates that 1AKO cells exhibit greater branching and complexity in the proximal dendrites as compared to the WT neurons.

Morphology in the juvenile mouse

Pyramidal cell dendrites in the juvenile mice were shorter in length than dendrites from the adult hippocampus. Total dendritic length was greater in P13 1AKO cells as compared to P13 WT cells (p<0.05, Figure 2D), similar to the pattern seen in the adult
mice. There were also a greater number of dendritic ends in P13 1AKO mice compared to P13 WT (p<0.05). The quantity of dendritic branches (Figure 2C) and total number of nodes did not differ between genotypes. A Sholl analysis showed that there was a main effect of genotype with 1AKO neurons having significantly more intersections (p<0.05) and greater dendritic length (p<0.05) than WT cells (Figure 2G). However, there was no interaction with radius.

*Evoked responses*

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum of CA1 before and after high-frequency tetanic stimulation of the Schaffer collaterals. The percent change of fEPSP slope from baseline, as averaged over the last 10min of the 60min post-tetanus interval, was 54.8±0.6% in hippocampal slices from adult WT mice (N=10) and 28.2±0.9% in slices from adult 1AKO mice (N=10, Figure 3). This demonstrated a significant deficit (p<0.0001) in CA1 LTP in the 1AKO mouse.

Evoked glutamatergic (eEPSC) and GABAergic (eIPSC) responses were obtained using whole-cell electrophysiology in order to further understand the mechanism underlying this LTP deficit in the 1AKO slices. The Schaffer collaterals were stimulated using increasing levels of current in order to obtain input-output curves for each cell. WT pyramidal cells (N=12) exhibited a significantly higher eEPSC response than 1AKO cells (N=15, p<0.0001, Figure 4A). This indicates an insufficiency in glutamatergic release or
receptors in the 1AKO hippocampus that may contribute to the deficit in LTP. Conversely, there were no differences in eIPSCs between 1AKO (N=16) and WT (N=14) cells, showing that GABA is not a contributing factor in the difference in LTP between genotypes (Figure 4B).

DISCUSSION

The current study aimed to characterize cellular properties in the hippocampus of the 1AKO mouse that may contribute to the behavioral phenotype of anxiety and cognitive deficits. Several main conclusions can be drawn from these experiments. The first is that genetic deletion of the 1AR resulted in increased CA1 pyramidal cell proximal dendritic branching. Since there were already significant morphological differences between the 1AKO and WT at P13, this change began to occur prior to the start of the critical period for the 1AKO behavioral phenotype. Secondly, pyramidal neurons of 1AKO mice received less glutamatergic input than those of WT mice. In the juvenile mouse, this was shown by decreased spontaneous frequency of excitatory events, indicating a pre-synaptic mechanism (Del Castillo and Katz, 1954; Redman, 1990; Stevens, 1993). In the adult mouse, the change in amplitude and kinetics of spontaneous EPSCs points towards alterations in postsynaptic receptor number and subunits. Finally, activation of the hippocampal circuitry through stimulation of the Schaffer collaterals revealed a deficit in LTP in the 1AKO mouse. Analysis of the evoked components showed that the AMPA-mediated response in 1AKO pyramidal cells was significantly
diminished compared to WT cells. This indicates that a decrease in the number of AMPA receptors may be the underlying mechanism behind the reduced LTP and smaller sEPSCs in the 1AKO.

*Morphology in adult and juvenile mice*

Pharmacological blockade of the 1AR during the third to fifth postnatal weeks has been shown to phenotypically copy the behavior of the 1AKO mouse (Lo Iacono *et al*, 2008; Tsetsenis *et al*, 2007). A recent study by Ferreira *et al* (2010) demonstrated that the same pharmacological treatment also resulted in increased dendritic arborization in the stratum radiatum (SR) of 1AKO mice in hippocampal pyramidal cells at P35. Using hippocampal cell cultures, they demonstrated that normal dendritic growth dynamics were a result of 5-HT acting through the 1AR to reduce actin polymerization and restrict growth cones. Our experiments add important data showing that genetic deletion of the 1AR produced increased proximal dendritic branching in the 1AKO that was already present at the beginning of the third postnatal week.

A mechanism occurring prior to the critical period for the 1AKO phenotype that leads to the differences in morphology seen at P13 therefore must be present. One possible explanation is increased serotonin efflux in the hippocampus. Several studies have looked at extracellular serotonin levels in the adult 1AKO mouse and have found no differences in ventral hippocampus (Knobelman *et al*, 2001b; Richardson-Jones *et al*, 2011). However, early postnatal WT mice show high serotonin levels in the
hippocampus that peak on day 14 (Mitchell et al, 1990). This may be due to a lack of functional coupling of the 1AR in animals before that time in spite of high expression levels of the receptor, a mechanism previously proposed based on studies in rat cortex (Beique et al, 2004). By the time the animal reaches adulthood, serotonin levels are greatly reduced, such that there is little tonic activation of 1ARs (Haddjeri et al, 2004). This may account for the time course of the critical period for the 1AKO (Ferreira et al, 2010a; Gross et al, 2002; Lo Iacono et al, 2008).

Cellular properties in adult and juvenile mice

In the first two weeks of postnatal development, cell signaling in the rodent hippocampus lacks an AMPA receptor-mediated component (Durand et al, 1996; Liao and Malinow, 1996; Wu et al, 1996). During this time, GABAergic transmission is excitatory and works in concert with NMDA receptor activation, allowing the depolarizing activity necessary for the formation of mature synapses (Ganguly et al, 2001; Leinekugel, 2003). Excitation in these immature neurons alters synaptic efficacy and strengthens connections in a Hebbian manner, much like in the adult (Ben-Ari, 2002). At age P13, 1AKO mice showed decreased frequency of spontaneous EPSCs, indicating that pyramidal cells received less excitatory input at this time. This data indicates that in the 1AKO mouse there was less synaptogenesis, which would lead to decreased synaptic activity in the adult 1AKO. In support of this conclusion, we showed that adult 1AKO mice have decreased sEPSC amplitude and smaller evoked AMPA-
mediated EPSCs compared to WT mice, demonstrating that the number of AMPA receptors was diminished in the 1AKO. Additionally, the kinetics of AMPA receptors were altered, as evidenced by increased rise and decay times in 1AKO pyramidal cells. These changes may be due to modifications in AMPA receptor subunit composition that occurred early in the development of the 1AKO mouse hippocampus.

Within SR, the number or density of AMPA receptors is the major contributing factor to synaptic strength (Nicholson et al., 2006). While the electrophysiology data indicated that AMPA receptor-mediated input in this region was reduced in the 1AKO mouse, the morphology of pyramidal cells showed that in the SR there was increased dendritic branching and complexity compared to WT mice. Unlike WT cells, patch-clamped pyramidal cells in the 1AKO did not demonstrate electrotonic filtering. Filtering occurs when events generated distally but recorded somatically are delayed and diminished due to the cable properties of the dendrites (Rall, 1969). There was no change in capacitance between 1AKO and WT neurons (results not shown), indicating that the larger membrane resistance of 1AKO cells is due to a decreased number of ion channels. Therefore 1AKO neurons would have less loss of current and thus decreased filtering. Additionally, this could indicate that synaptic events in the 1AKO may be generated closer to the cell body within the complex dendritic arborization.

Decreased synaptogenesis in the 1AKO would also lead to an overall reduction in the number of ion channels. CA1 pyramidal cells in the adult 1AKO mouse showed greater resistance and tau, both of which contribute to the increased excitability seen in the adult, but not the P13 frequency-intensity plots. These altered characteristics could
be reflective of an increase in the surface area of the cell, stemming from the larger dendritic branching, and/or from the reduction in the number of ion channels due to decreased synaptogenesis. Additionally, the smaller activation gap in 1AKO cells would contribute to the greater intrinsic excitability, which is independent of synaptic input. These differences in membrane properties were not present in the juvenile 1AKO mouse, indicating that changes in morphology occur concurrently with and may drive the reduction in the number of channels and modifications in cellular properties seen in the adult.

Long-term potentiation

LTP is a phenomenon by which synaptic strength between pre- and post-synaptic neurons is increased. The most commonly studied type of LTP at the CA3-CA1 synapse is NMDA receptor-dependent. However, there is evidence that postsynaptic modification in CA1 may occur based on AMPA receptor recruitment (Malinow and Malenka, 2002). Here we found that 1AKO mice exhibited a deficit in CA1 LTP. Both spontaneous and evoked EPSCs in the 1AKO reflected a decrease in amplitude of glutamatergic signaling likely due to a decreased number or density of postsynaptic AMPA receptors. Furthermore, the kinetics of sEPSCs indicate possible changes in AMPA receptor subunits. AMPA subunits are known to affect the trafficking properties and functionality of the receptor, and phosphorylation of the GluR1 subunit is required for synaptic plasticity and the retention of spatial memory (Derkach et al, 2007; Lee et al, 2003).
Therefore, decreased number and activity of AMPA receptors may underlie the deficit in LTP in the 1AKO mouse hippocampus. As memories are thought to be encoded by synaptic strength, this also presents a mechanism that explains the hippocampal-dependent deficits in spatial learning and memory that have previously been shown in the 1AKO mouse (Bliss and Collingridge, 1993; Sarnyai et al, 2000).

Conclusion

There have been few electrophysiological studies examining the hippocampus of the 1AKO mouse in adulthood and during the critical period for the development of the behavioral phenotype. Previous work from our laboratory shows correlations between high glutamatergic activity and anxiety-like behavior in mice. However, in the current study, electrophysiological and morphological comparisons were made between the two genotypically verified groups. Juvenile 1AKO mice have decreased glutamatergic input and increased dendritic branching, suggesting that synaptogenesis and cell architecture drive changes during the critical period, possibly due to increased serotonin efflux from the raphe. Additionally, these experiments show decreased AMPA-mediated responses in the adult 1AKO that likely underlie the smaller LTP response and learning and memory deficits seen in these mice. These findings provide further evidence for the role of the 1AR in the development of mood and cognitive disorders.

ACKNOWLEDGEMENTS
We thank Mark Geyer for providing the founders for the colony of mice used in these experiments, Akiva Cohen for critical advice on LTP experiments, and Zachary Spangler for animal husbandry and genotyping.
FIGURE LEGENDS

Figure 1. Frequency-intensity plots of the frequency of AP firing rate in response to increasing depolarizing current pulses in neurons recorded from WT and 1AKO mice. (A) Representative data traces in current-clamp (top) used to generate frequency-intensity plots (bottom), scale bar: 20 mV, 50 ms. P13 WT cells (N=20) did not differ in excitability from 1AKO (N=14). (B) Adult WT cells (N=26) show significantly more excitability as compared to 1AKO cells (N=28). *p<0.05
Figure 1

A

WT

1AKO

Intensity (pA)

AP frequency (Hz)

B

WT

1AKO

Intensity (pA)

AP frequency (Hz)
Figure 2: Morphological analyses of CA1 pyramidal neurons in WT and 1AKO. (A) Representative traces of neurons from adult WT (left, N=19) and 1AKO (right, N=24) cells, and (B) from P13 WT (left, N=11) and 1AKO (right, N=9) cells. Scale bars represent 100μ. (C) Number of dendritic branches differs between WT and 1AKO in the adult but not the juvenile neurons. (D) Total dendrite length represents the average sum of all dendritic processes. 1AKO neurons had longer dendrites in total than WT cells in both the adult and P13 animals. (E) Schematic of representative neuron used for Sholl analysis. (F) Dendrites in the adult 1AKO showed significantly more complexity as compared to WT dendrites. This increase in branching took place in proximal dendrites from 60-180μ from the soma. (G) Dendrites of P13 1AKO cells had a greater overall number of intersections as compared to WT, although the interaction with radius was not significant. *p<0.05
Figure 2

(A) WT, (B) KO, (C) # of primary dendrites, (D) Total dendritic length, (E) Distance from soma (# of intersections), (F) WT, (G) 1AKO
Figure 3: LTP in adult WT and 1AKO mouse hippocampal slices. (A) Representative fEPSP traces from WT and 1AKO slices averaged over a 30 min baseline (black) and for 60 min after tetanic stimulation (gray). Scale bar: 0.25mV, 5ms. (B) High frequency tetanic stimulation (4 x 100Hz, shown by the arrow) resulted in an increase in slope of the fEPSP that was significantly greater in WT (N=10) as compared to 1AKO slices (N=10).
Figure 3

A

B
Figure 4: Evoked post-synaptic currents in WT and 1AKO CA1 pyramidal cells from adult mice, expressed as percentage of the highest current used to stimulate the Schaffer collaterals in order to produce a maximal response. (A) Representative traces of eEPSCs (top, scale bars = 200pA, 10ms). eEPSCs were significantly greater in the WT neurons (bottom, N=12) as compared to the 1AKO cells (N=15, p<0.0001). (B) There were no differences in eIPSCs between the genotypes. *p<0.05
Figure 4

A

B

WT
1AKO

% max stimulus

0 20 40 60 80 100

-1250 -1000 -750 -500 -250 0

eEPSC (pA)

% max stimulus

0 20 40 60 80 100

-500 -400 -300 -200 -100 0

dIPSC (pA)
TABLE LEGENDS

Table 1. Cellular characteristics of CA1 pyramidal cell neurons recorded from adult and juvenile WT and 1AKO mice. Values are shown as mean ± SEM, *p<0.05 unpaired Student’s t-test between WT and 1AKO.
Table 1

<table>
<thead>
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<th>Cell type</th>
<th>RMP (mV)</th>
<th>Res (MΩ)</th>
<th>Tau (ms)</th>
<th>AP threshold (mV)</th>
<th>Activation gap (mV)</th>
<th>AP amplitude (mV)</th>
<th>AP duration (ms)</th>
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<td>-63.3±0.8</td>
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<td>19.3±0.7</td>
<td>-42.5±0.9</td>
<td>20.8±0.8</td>
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<td>1AKO (N=28)</td>
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Table 2. Spontaneous post-synaptic currents from pyramidal cell neurons recorded from WT and 1AKO mice. Values are shown as mean ± SEM, *p<0.05 unpaired Student’s t-test between WT and 1AKO.
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<th>Amplitude (mV)</th>
<th>Rise time (ms)</th>
<th>Decay time (ms)</th>
<th>Charge</th>
<th>Phasic current</th>
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<td>1AKO sIPSC (N=17)</td>
<td>10.7±1.7</td>
<td>14.2±1.7</td>
<td>2.6±0.1*</td>
<td>9.6±1.4 slow</td>
<td>134.0±8.9</td>
<td>1529.3±285.0</td>
</tr>
<tr>
<td>P13 WT sEPSC (N=20)</td>
<td>1.3±0.2</td>
<td>10.5±0.8</td>
<td>3.5±0.1</td>
<td>5.6±0.3</td>
<td>78.3±7.4</td>
<td>108.1±20.0</td>
</tr>
<tr>
<td>P13 1AKO sEPSC (N=14)</td>
<td>0.8±0.1*</td>
<td>9.6±0.5</td>
<td>3.2±0.1</td>
<td>6.1±0.5</td>
<td>74.2±3.9</td>
<td>58.9±5.8</td>
</tr>
</tbody>
</table>
CHAPTER 3:

CONCLUSIONS AND FUTURE DIRECTIONS
The preceding chapters describe my work characterizing the physiological and structural properties of pyramidal cell neurons within the CA1 region of the hippocampus in the 1AKO mouse. Previous research has shown that the 1AKO mouse has a phenotype of behavioral anxiety and cognitive deficits in learning and memory; however, a causative link between receptor and behavior has yet to be established (Heisler et al., 1998; Klemenhagen et al., 2006c; Parks et al., 1998; Ramboz et al., 1998; Sarnyai et al., 2000). Although the 1AR has been associated with clinical anxiety, there are obvious barriers to studying brain activity on the cellular level in the human population (Drevets et al., 1999; Lesch et al., 1992; Meltzer et al., 1995; Sargent et al., 2000; Savitz et al., 2009; Truchot et al., 2008). The overall goal of this research was to elucidate the cellular mechanisms behind the behavioral traits seen in the 1AKO. While anxiety and cognition certainly involve multiple brain regions, the hippocampus has been implicated in the etiology of the behavioral phenotype of the 1AKO mouse (Ferreira et al., 2010a; Gross et al., 2002; Lo Iacono et al., 2008). It was hypothesized that genetic deletion of the 1AR during a critical period in development would affect hippocampal circuitry, with possible implications for cell signaling within the glutamate, GABA, and CRF neurotransmitter systems.

The work presented in Chapter 2 demonstrated a cellular mechanism by which learning and memory deficits in the 1AKO mouse may develop. Results of this study ascertained a diminished LTP response in the 1AKO mouse compared to the WT, with evidence pointing towards a decrease in the activation of AMPA receptors as the cause. Furthermore, this research suggests a developmental process that could have resulted in
the establishment of the LTP and AMPA deficit. As LTP is commonly thought of as a cellular mechanism of memory formation, these findings support previous research showing learning and memory deficits in the 1AKO (Bliss et al., 1993; Sarnyai et al., 2000). Previous studies have also shown AMPA trafficking to underlie the consolidation of aversive memories, indicating that regulation of AMPA expression in the hippocampus affects the formation of emotional memory (Hu et al., 2007; Mitsushima et al., 2011; Thoeringer et al., 2011; Whitlock et al., 2006). The developmental time course of the proposed mechanism coincides with the critical window for the behavioral effects of the 1AR deletion on the 1AKO mouse (Ferreira et al., 2010a; Gross et al., 2002; Lo Iacono et al., 2008).

The highest expression of CRF in the rodent hippocampus coincides temporally with the critical period for development of anxiety-like behavior in the 1AKO (Chen et al., 2001; Gross et al., 2002; Lo Iacono et al., 2008). In the Appendix, I show that the cognitive impairments in the 1AKO mouse involve activation of CRF₁ receptors. CRF has been shown to decrease hippocampal population spikes and prevent the occurrence of LTP in the hippocampus (Rebaudo et al., 2001). Several studies have shown that chronic stress in the rodent decreases hippocampal plasticity and impairs memory through a CRF₁ receptor-mediated mechanism that can be reversed by CRF₁ antagonism (Chen et al., 2004b; Heinrichs et al., 1996; Ivy et al., 2010; Joels et al., 2004). Similar to our results, Ivy et al. (2010) reported that chronic stress early in the life of the rat results in an increased number of CRF-expressing interneurons in CA1, attenuated LTP, and behavioral learning and memory impairments in the adult animal compared to controls.
Furthermore, all of these effects are blocked with a CRF₁ antagonist administered after the chronic stress paradigm. Taken together, this could suggest that 1AR deficiency and early-life stress have a common pathway, i.e. excessive activation of CRF₁ receptors. However, although we and others have shown that 5-HT₁A blockade or deletion results in increased pyramidal cell dendritic arborization, many studies have shown dendritic atrophy resulting from chronic stress or application of CRF, indicating divergent mechanisms or effectors (Chen et al., 2004a; Ferreira et al., 2010a; Ivy et al., 2010; Magarinos and McEwen, 1995; McLaughlin et al., 2007; Watanabe et al., 1992). Future studies will be needed to characterize the underlying differences between these models.

One possibility for the inconsistencies between these two models is disparate effects of 5-HT or CRF on other of their respective receptor subtypes present within the hippocampus. Additionally, our studies do not suggest a time course for the upregulation of CRF-containing interneurons in CA1 or increased activation of CRF₁ receptors. Future studies could aim at distinguishing the developmental progression by measuring the number of CRF interneurons at different time points, or using microdialysis to examine the level of extracellular CRF in the hippocampus. Although it is possible that levels of 5-HT are also altered in the dorsal hippocampus of the 1AKO, several studies looking at ventral hippocampus in the adult have found no significant difference (Knobelman et al., 2001a; Richardson-Jones et al., 2011). However, it has been reported that extracellular levels of 5-HT are low in general in the adult rodent brain such that there is little tonic activation of 1ARs (Haddjeri et al., 2004). On the other hand juvenile WT mice show high serotonin levels in the hippocampus that peak on day 14 (Mitchell et
al, 1990). This may account for the defined critical period for the 1AKO phenotype (Ferreira et al, 2010a; Gross et al, 2002; Lo Iacono et al, 2008). A comparison using microdialysis in the young 1AKO and WT would help to show whether 5-HT levels in the hippocampus are altered during this critical period.

Another area of future research suggested by these studies is whether the data presented in this thesis will relate back to the behavioral anxiety and cognitive deficits in the 1AKO mouse. For example, a study involving the effects of the CRF$_1$ receptor antagonist should be done to demonstrate whether it can rescue hippocampal-dependent learning and memory deficits in the 1AKO mouse. This study could comprise several factors, such as whether the CRF$_1$ antagonist needs to be administered centrally or systemically, and if it can affect the adult behavior by being given to the juvenile 1AKO mouse. Further electrophysiological experiments might be done in order to show whether CRF applied to hippocampal slices of WT mice can mimic the LTP deficit seen in slices from 1AKO mice. However, given the increase in CRF-containing neurons from the 1AKO hippocampus, it is possible that acute CRF administration in the WT mouse would not be able to simulate the physiology of the 1AKO mouse. There is evidence that transient CRF facilitates memory in the rodent, whereas prolonged exposure impairs memory (Heinrichs et al, 1996; Lee et al, 1993; Radulovic et al, 1999; Row and Dohanich, 2008).

Since these studies indicate that both a decreased number of AMPA receptors and excessive activation of CRF$_1$ receptors are involved in the learning and memory deficiencies of the 1AKO mouse, it is possible that one has a direct effect on the other. A
recent study determined that CRF affects fear memory consolidation via the stabilization of surface expression of GluR1-containing AMPA receptors in the hippocampus (Thoeringer et al., 2011). More studies will be needed to determine what if any role this plays in the LTP and memory deficits in the 1AKO mouse.

The data presented in this thesis support the hypothesis that genetic deletion of the 1AR alters neural circuitry during development to produce the anxiety and cognitive deficits seen in the adult animal. With this work I add to the body of knowledge on the role of the 1AR in clinical anxiety disorders. Furthermore, the 1AR has been shown to have a function in the cognitive problems associated with Alzheimer’s disease and dementia (Kepe et al., 2006; Schechter et al., 2002; Truchot et al., 2007). In addition to a model for cognitive deficits in anxiety, the 1AKO may also be beneficial in research on these diseases. Although it is likely that the etiologies are different, the similarities may be beneficial in elucidating novel targets for therapeutic intervention.

Constitutive genetic deletion has several limitations, and conditional knockouts are being generated to separate spatial and temporal aspects of findings found in the 1AKO (Richardson-Jones et al., 2010; Richardson-Jones et al., 2011). Given the complexity of human anxiety and cognitive disorders, it is difficult to predict which conditions will best model the complicated genetic components of these diseases. Elucidating the neurological basis for behavior in an animal model provides a deeper understanding of the neurobiological mechanisms involved in the development of human disorders. The currently available medications for anxiety disorders leave many patients without adequate treatment or vulnerable to relapse. Clinical studies guide what
researchers look for in an animal model. Future research using models of illness such as the 1AKO should aid in the design of better therapeutics to mitigate or prevent the pathological development of anxiety.
APPENDIX:

5-HT1A DELETION ALTERS HIPPOCAMPAL CRF EXPRESSION AND SYNAPTIC PLASTICITY

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ABSTRACT

Mice in which the serotonin 1A receptor (1AR) has been genetically deleted (1AKO) show anxiety-like behavior and deficits in learning and memory. These behaviors depend on 1AR expression within the hippocampus during a critical time period during development. Interneurons containing corticotropin-releasing factor (CRF) peak during the same time period and likely play a role in the development of 1AKO aberrant behavior. This study used in vitro electrophysiology and immunohistochemistry to examine CRF₁ receptor-mediated effects in the CA1 region of the hippocampus. We found that in the 1AKO mouse, the number of CRF-containing interneurons was increased. Administration of CRF peptide had similar effects in the 1AKO and WT pyramidal cells, however CRF₁ receptor antagonism reversed the 1AKO deficit in LTP. Together these data indicate that an elevated level of extracellular CRF hippocampus contributes to hippocampal dysfunction and may underlie the learning and memory deficits in the 1AKO.
INTRODUCTION

Human studies link the serotonin (5-HT) 1A receptor (1AR) with mood disorders, such as depression and anxiety, and deficits in cognitive functioning (Borg, 2008; Lesch et al, 1992; Savitz et al, 2009). These disorders often display a high incidence of comorbidity, although whether this connection is correlative or causal has not yet been determined (Gotlib and Joormann, 2010). Mice that have the 1AR genetically deleted, or knocked out (1AKO), throughout life show anxiety-like behavior and deficits in learning and memory, and may therefore be a good model for human mood and cognitive disorders (Heisler et al, 1998; Klemenhagen et al, 2006c; Parks et al, 1998; Ramboz et al, 1998; Sarnyai et al, 2000).

1ARs are located on 5-HT neurons in the raphe as autoreceptors and in areas throughout the brain that are innervated by raphe neurons postsynaptically as heteroreceptors. Studies using selective genetic deletion or pharmacological agents reveal diverse spatial and temporal roles for populations of 1ARs. Peripheral 1AR antagonism fails to produce anxiety-like behavior in the adult mouse (Cao et al, 1997; Fletcher et al, 1996). However, pharmacological blockade in the juvenile showed that 1AR activity is necessary from postnatal days P13-P34 for normal behavior, revealing a critical time window for the development of anxiety-like behavior (Lo Iacono et al, 2008). Lifetime genetic elimination of the autoreceptor produces a mouse that has an anxious phenotype, whereas knockout of the heteroreceptor leads to a non-anxious mouse that exhibits a depression-like phenotype (Richardson-Jones et al, 2010; Richardson-Jones et al, 2011). Of regions innervated by 5-HT neurons, the 1AR exhibits the highest
expression in the CA1 region of the hippocampus (Chalmers et al, 1991; Gross et al, 2002). 1AKO mice show deficits in hippocampal-mediated learning and memory tasks, but not in memory tests that are mediated by other brain regions (Sarnyai et al, 2000). Administration of a 1AR agonist into the dorsal hippocampus produces an anxiolytic effect, but this effect does not occur when administered to amygdala or septum (Menard and Treit, 1999). These experiments demonstrate that the hippocampus is crucial to the formation of the behavioral phenotype in the 1AKO.

In the CA1 region of the hippocampus, GABAergic interneurons within stratum pyramidale (SP) play an important role in the synchronization and timing of adult hippocampal pyramidal cell firing (Cobb et al, 1995; Sik et al, 1995; Ylinen et al, 1995). One type of interneuron contains corticotropin-releasing factor (CRF) and is known to peak in number in the rodent between P11-P18 (Chen et al, 2001; Danglot et al, 2006). During the first two postnatal weeks, GABA is excitatory and GABA\textsubscript{A} mediated depolarization is necessary for the development of new synapses, the activation of NMDA receptors, the stabilization of AMPA receptors, and the formation of mature neuronal circuits (Ben-Ari, 2001; Ben-Ari et al, 1997; Kasyanov et al, 2004). Therefore, interneurons are poised to exert great influence over neural circuitry in this developmental period, which coincides with the critical period for the development of anxiety, i.e. P13-P34.

The CRF neuropeptide is a key mediator in the neuroendocrine response to stress, but also acts as an excitatory neuromodulator in stress-associated limbic regions such as the hippocampus (Aldenhoff et al, 1983; Baram and Hatalski, 1998; Brunson et al, 2002;
Smith and Dudek, 1994; Vale et al, 1981). While CRF is located in somata, axons, and boutons of GABAergic interneurons, the CRF$_1$ receptor is found on pyramidal cell dendritic spines, and these interneurons are in a position to directly influence pyramidal cell excitability via reciprocal interactions (Chen et al, 2004b). Application of CRF is known to increase the frequency of pyramidal cell spontaneous discharge, reduce the size of afterhyperpolarizations (AHPs), and depolarize CA1 pyramidal neurons (Aldenhoff et al, 1983; Blank et al, 2003; Hollrigel et al, 1998).

CRF has age-specific effects, with juvenile animals being more sensitive to the compound (Smith et al, 1994). Administration of CRF to juvenile rats evokes limbic seizure activity more rapidly and at lower doses than in the adult rodent (Baram and Ribak, 1995; Baram and Schultz, 1991; Smith et al, 1994). In lower doses, CRF administered to the juvenile rodent induces hippocampal neuronal injury and causes hippocampal-mediated learning and memory impairments throughout the life of the animal, similar to those induced by early-life stress (Baram et al, 1995; Baram et al, 1991; Brunson et al, 2001; Ehlers et al, 1983; Marrosu et al, 1988; Sanchez et al, 1998). However, equivalent doses in the adult rat had less or no effect. Since neural activity may play a role in the regulation of neuropeptide levels, increased excitability of 1AKO CA1 neurons due to lack of inhibition by 1AR may cause lifetime increases in baseline CRF levels (Gall et al, 1990). In support of this, administration of CRF early in life leads to upregulation of hippocampal CRF$_1$ receptor expression that persists into adulthood (Brunson et al, 2001).
Although much is known about the CA1 pyramidal-interneuron circuit in the adult wild-type mouse, there have been few studies that looked at how this circuit is affected in the 1AKO mouse, and how absence of the 1AR during the critical period leads to the development of anxiety-like behavior and learning and memory deficits. This study uses immunohistochemistry and electrophysiology to show how the number of CRF-containing interneurons and activity of hippocampal pyramidal cells is altered in the juvenile and adult 1AKO mouse.

MATERIALS AND METHODS

Animals

Founders from an established colony in the laboratory of Dr. Mark Geyer, University of California, San Diego, CA were obtained to generate a colony at Children’s Hospital of Philadelphia. Male offspring from heterozygous pairings were used in this study. Litters were weaned, separated by sex, and genotyped at 3 weeks of age. For all experiments, adult (2-5 months) and juvenile (12-14 postnatal days) homozygous wild-type (WT) and knockout (1AKO) male mice on a 129/Sv background (for details, see (Ramboz et al, 1998)) were used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional IACUC committee.
Electrophysiology

Brain slices were prepared as previously described (Richardson-Jones *et al.*, 2011; Tsetsenis *et al.*, 2007). Briefly, mice were decapitated and their brains dissected out in cold artificial cerebrospinal fluid (aCSF) in which NaCl was replaced with sucrose (248 mM). The forebrain was blocked and cut on a Leica VT1000s vibratome (Leica Microsystems, Bannockburn, IL). Coronal slices (200 μm thick for whole-cell patch-clamp, 400 μm for field recordings) containing hippocampus were placed in aCSF (in mM, 124 NaCl, 3.2 KCl, 1.25 NaH$_2$PO$_4$, 1.3 MgSO$_4$, 2.5 CaCl$_2$, 10 dextrose and 26 NaHCO$_3$) at 37 °C bubbled with 95% O$_2$/5% CO$_2$. After one hour, slices were kept at room temperature. Individual slices were placed in a recording chamber and perfused with aCSF at 2 ml/min at 32 °C maintained by an in-line solution heater (TC-324, Warner Instruments). Neurons were visualized using a Nikon E600 upright microscope, and signals were collected and stored using a Multiclamp 700B, Digidata 1322A analog-to-digital converter, and pClamp 9.0 software (Molecular Devices, Sunnyvale, CA).

Whole-cell recordings were obtained using electrodes filled with an intracellular solution of (in mM) 130 K-gluconate, 5 NaCl, 10 Na phosphocreatine, 1 MgCl$_2$, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.5 Na$_2$GTP, pH 7.3. For evoked PSCs, a cut was made between CA3 and CA1 to prevent recurrent excitation. CA1 pyramidal neurons were patched and cell characteristics recorded using current clamp techniques as previously described (Beck *et al.*, 2004). Voltage clamp recordings were conducted holding the membrane potential at -60 mV (Lemos *et al.*, 2011). To isolate AMPA-mediated eEPSCs, bicuculline methiodide (BMI, 20 mM, Sigma-Aldrich, Saint Louis,
MO) and (2R)-amino-5-phosphonopentanoate (AP5, 30mM, Sigma-Aldrich) were added to the superfusing aCSF. GABAA-mediated eIPSCs were isolated similarly with the addition of AP5 and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20mM, Sigma-Aldrich). A subset of cells received bath exposure to ovine CRF (gift from Wylie Vale, Salk Institute, San Diego, CA) or the CRF₁ antagonist CP-154526 (300nM, Tocris Bioscience, Ellisville, MO). Preliminary experiments yielded a maximal response to CRF at a concentration of 100nM (data not shown). All drugs were made in stock solutions, diluted on the day of the experiment, and added directly to the superfusing aCSF.

For extracellular evoked potential recording, glass electrodes were filled with 4M NaCl and placed in stratum radiatum of area CA1. The Schaffer collateral afferent pathway was stimulated with a concentric bipolar platinum/iridium electrode (FHC, Bowdoin, ME) and field excitatory postsynaptic potentials (fEPSPs) were recorded. Input/output curves from 0.001 to 1 mA were generated and the stimulation that produced half maximal response was used for the rest of the experiment. Baseline potentials were monitored for 30 min before tetanic stimulation. Long-term potentiation (LTP) was induced by four trains of conditioning stimulation (1s at 100 Hz) at 30-s intervals (high-frequency stimulation (HFS)). Responses were subsequently followed for 60 min at 20-s intervals.

_Diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry_
Adult animals underwent adrenalectomy (ADX) at Children’s Hospital of Philadelphia and were given access to a 0.9% saline solution to help maintain sodium chloride balance. 7-8 days later, mice were deeply anesthetized, then perfused transcardially with 20 ml saline followed by 50 ml 4% paraformaldehyde. The brains were isolated, postfixed in paraformaldehyde for two hours at 4°C, then submerged in 30% sucrose in 0.1 M phosphate buffer. Coronal sections encompassing the dorsal hippocampus were cut on a cryostat into 40-μm-thick sections. Endogenous peroxidase activity was quenched in 0.75% H2O2 made in phosphate-buffered saline solution (PBS; pH 7.4), then slices were washed in PBS with 0.25% Triton X-100 and 0.5% bovine serum albumin (PBST-BSA) and incubated in rabbit anti-CRF (1:6000; gift from Wylie Vale) for three nights at 4°C. After several washes, sections were incubated in biotinylated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) for 90 min at room temperature. The sections were then rinsed with PBS and incubated with the Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) at room temperature for 90 min. Finally, the colored reaction product was developed using the Vectastain DAB peroxidase substrate kit with nickel solution (Vector). Control experiments that omitted primary antibody yielded no staining (data not shown). After washes in 0.1M PB, sections were mounted on Superfrost slides and coverslipped with Permount mounting media (Southern Biotech, Birmingham, AL). Labeled cells were visualized under a bright-field light microscope. Images were captured using a digital camera and Leica Application Suite software (Leica Microsystems Inc., Buffalo Grove, IL). Slices containing only dorsal hippocampus were subdivided into areas CA1 and CA3. The border was designated using the abrupt change in CRF-positive neuropil
staining of the stratum pyramidale, which is thicker in CA3. Cells were counted in each hemisphere separately, from the CA3-CA1 border to the apex of CA1, without knowledge of genotype.

Data analysis

MiniAnalysis software (Synaptosoft, Inc., Decatur, GA) was used to analyze spontaneous and miniature EPSC events for frequency, amplitude, rise time, decay time, baseline holding current, and area (Crawford et al, 2011; Lemos et al, 2011). The slope and amplitude of fEPSP and ePSCs were measured using pClamp 9 software. fEPSP was expressed as percent of pre-tetanus baseline values, and ePSCs expressed as percent of maximal stimulus before an action potential was elicited. LTP was quantified as the mean of the last 10 min of 60 min post-tetanus. Statistica (StatSoft, Tulsa, OK) or Prism (GraphPad, La Jolla, CA) software were used for all statistical tests. Passive and active membrane characteristics were analyzed using Clampfit software (Molecular Devices) and then compared between WT and 1AKO, pre- and post-drug using repeated measures ANOVA followed by Tukey post-hoc analysis. The number of CRF-containing neurons counted for each hippocampal slice was compared between WT and 1AKO using unpaired Student’s t-test. Repeated measures ANOVA was used to examine frequency-intensity (F-I) plot and all evoked responses, followed by Tukey post-hoc analysis. Significance levels were set at 0.05.
RESULTS

CRF-containing cells were stained with DAB to assess differences in expression within the pyramidal cell layer of CA1 (Figure 1A). The number of CRF cells per slice was significantly greater in 1AKO (10.19 ± 0.5, N=188 slices from 6 mice, p<0.0001) compared to WT (7.76 ± 0.4, N=144 slices from 5 mice) using unpaired Student’s t-test with Welch’s correction for unequal variance (Figure 1B).

To understand how increased CRF expression within the CA1 pyramidal cell layer might affect cellular properties and cell signaling, pyramidal neurons were recorded using whole-cell patch-clamp electrophysiology. There were no baseline differences in passive or active cell membrane characteristics between 1AKO (N=13) and WT (N=14) neurons, except for a main effect of genotype on the frequency-intensity (F-I) plot, showing that 1AKO neurons tend to be more intrinsically excitable (p<0.05, Figure 2B). Administration of the moderately selective CRF₁ agonist ovine CRF (100nM) produced a depolarization of the membrane potential in cells from both genotypes, but a reduction in membrane resistance only in WT neurons (p<0.05, Figure 2A). The depolarization resulted in a decrease in the activation gap, defined as the difference between resting membrane potential and threshold for action potential, in both 1AKO and WT cells (p<0.0005). The action potential duration was also longer in both genotypes after CRF administration. CRF did not produce any differences in the F-I plot (Figure 2B).

Mice were also recorded at age P13. This age was chosen because it is the beginning of the critical time period for the development of anxiety-like behavior in the
1AKO mouse (Lo Iacono et al, 2008). At baseline, there were no differences in cell characteristics between 1AKO (N=14) and WT (N=20) cells. However, analysis of synaptic activity showed that 1AKO cells (0.81 ± 0.08 Hz) had decreased frequency of spontaneous excitatory post-synaptic currents (sEPSCs) compared to WT (1.26 ± 0.16 Hz, p<0.05), indicating 1AKO CA1 neurons receive less excitatory input at P13. Like in the adult hippocampus, application of CRF produced a depolarization of the membrane potential in both 1AKO and WT cells (p<0.00001), but a decrease in resistance only in WT (Figure 2D, p<0.005). There were no differences between 1AKO and WT neurons in their intrinsic excitability, as measured by the F-I plot, either before or after CRF (Figure 2C). CRF decreased the action potential threshold across genotype (p<0.00001), and therefore reduced the activation gap in both WT and 1AKO cells (p<0.005). There was also an increase in action potential duration across genotype, similar to the adult hippocampal neurons (p<0.005). Unlike what was seen in the adult, CRF increased the sEPSC frequency across genotype (p<0.0005). Finally, CRF increased the total phasic current, measured as the sEPSC frequency multiplied by the area under the curve, across genotype (p<0.05).

While these experiments looked at the passive and active properties of individual CA1 pyramidal neurons in hippocampal slices at baseline conditions, it remained a question what effects CRF may have in an activated neuronal system. LTP was used because it is considered to be a major cellular mechanism that underlies learning and memory. 1AKO mice have been shown to be deficient in learning and memory tasks. In a previous experiment from our lab, hippocampal slices from 1AKO and WT mice (N=10
each) were given a high-frequency tetanus via stimulation of the Schaffer collaterals after a stable baseline was established. Field excitatory post-synaptic potentials (fEPSPs, Figure 3A) that were subsequently followed for 60 min displayed an increase in slope of 54.46 ± 14.9% for WT slices and 28.14 ± 8.9% for 1AKO slices over the last 10 min, showing that in the 1AKO CA1 region of the hippocampus there was a deficit in LTP (p<0.00001, Figure 3B). In a separate group of mice for this study (9 1AKO, 9 WT slices), the CRF<sub>1</sub> antagonist CP-154526 was bath applied at 300nM after a 15 min baseline and 30 min prior to tetanus (Figure 3D). CP-154526 application by itself resulted in a 13.06 ± 5.6% slope increase in WT hippocampus and a 15.54 ± 4.1% increase in 1AKO hippocampus compared to baseline (not shown). High-frequency tetanus further augmented fEPSP slope in both WT (47.53 ± 11.3%) and 1AKO slices (45.24 ± 9.9%, WT vs. 1AKO p=0.206), demonstrating that pharmacological blockade of CRF<sub>1</sub> receptors was able to reverse the deficit in 1AKO LTP. There was no significant effect of CP-154526 on % LTP in WT slices, indicating that the effect of CRF<sub>1</sub> blockade on LTP was confined to 1AKO hippocampus.

A previous experiment from our lab using whole-cell electrophysiology showed that the 1AKO had a deficit in AMPA receptor-mediated evoked EPSCs (Figure 4A) that may underlie the insufficient response to LTP. Based on the response to CP-154526 in the 1AKO hippocampus, we hypothesized that CRF<sub>1</sub> blockade might augment AMPA-mediated eEPSCs in 1AKO pyramidal cells. However, unlike the prior experiment, WT and 1AKO neurons (N=16 each) showed no difference in AMPAergic eEPSC responses
(Figure 4B, p=0.919). Furthermore, when CP-154526 was applied, there was no significant change in response in either 1AKO or WT cells.

**DISCUSSION**

The current study demonstrated that genetic deletion of the 5-HT1A receptor resulted in abnormalities in hippocampal circuitry and function. The major findings were that (1) juvenile and adult pyramidal cells showed a similar response pattern to CRF administration, (2) there were a greater number of CRF-containing interneurons in adult 1AKO compared to WT mice, and (3) the 1AKO hippocampus showed a diminished CA1 LTP that was reversed with CRF₁ blockade.

In these experiments, exogenously applied CRF had multiple effects on pyramidal cell membrane characteristics and synaptic activity in the adult and juvenile mouse hippocampus. Consistent with previous studies, CRF application caused adult pyramidal cells to depolarize, indicating increased excitability (Blank *et al.*, 2003; Hollrigel *et al.*, 1998). However, resistance was decreased only in WT pyramidal cells, and not in those of 1AKO mice. The concurrent increase in action potential duration in both genotypes indicates that these results may be due to an effect of CRF₁ receptor activation on K⁺ channels or the NA+/K⁺ pump. The same pattern of depolarization and resistance decrease in WT mice was observed in the juvenile mice, showing that the mechanism CRF effect on cellular properties has been established by the beginning of the critical period.
CRF\textsubscript{1} receptors are located preferentially on dendritic spines of pyramidal cells in the CA1 region of the hippocampus. This indicates that in the adult mouse, CRF may need to travel a considerable distance to receptors after being released from GABAergic terminals of basket cells, and that synaptic interaction is through volume transmission (Chen \textit{et al.}, 2004b). Therefore, it may be only during times of extensive excitation, such as during stress, that enough CRF peptide reaches the receptors to enhance synaptic plasticity (Chen \textit{et al.}, 2004a). In the young mouse, however, these GABAergic terminals of interneurons synapse directly onto pyramidal cell dendrites, suggesting that CRF exerts a greater influence during development through its proximity to CRF\textsubscript{1} receptors. Many studies have shown evidence that juvenile rodents do show increased sensitivity to CRF, for example CRF-induced limbic seizures and neuronal injury are more extensive in the young rat compared to the adult (Baram \textit{et al.}, 1991; Ehlers \textit{et al.}, 1983; Marrosu \textit{et al.}, 1988; Smith \textit{et al.}, 1994).

Furthermore, interneurons are known to express 1ARs during this postnatal period (Aznar \textit{et al.}, 2003). Without 1ARs to inhibit CRF-containing basket cells during the critical period, it is possible that CRF is released in greater quantities. Neural activity can regulate neuropeptide levels, and increased CRF\textsubscript{1} receptor binding during development may lead to upregulation of CRF and CRF\textsubscript{1} mRNA, resulting in upregulation of endogenous CRF peptide levels and/or CRF\textsubscript{1} receptor expression in the adult (Brunson \textit{et al.}, 2001; Gall \textit{et al.}, 1990). Therefore, although the effects of exogenous CRF administration were similar in the 1AKO and WT pyramidal cells at P13, it is possible
that endogenous CRF and CRF₁ receptor expression levels were upregulated in the adult 1AKO.

The current study used immunohistochemistry to uncover possible changes in CRF expression in the 1AKO hippocampus. We found that there were a greater number of CRF-containing interneurons in the CA1 pyramidal cell layer of the 1AKO. Interestingly, a similar effect has been shown in rat hippocampus using a chronic early-life stress paradigm (Ivy et al., 2010). However, the mechanism and timeframe by which these changes occur remains unknown. Cell counts in the juvenile 1AKO would help discern whether the increase in CRF-containing cells is altered before, during, or at the end of the critical period. Additionally, microdialysis could be used to determine whether the level of extracellular CRF in the hippocampus was increased in the juvenile and adult.

A previous study by this lab showed that LTP at the CA3-CA1 synapse is diminished in 1AKO hippocampal slices (unpublished). LTP is commonly considered to be the underlying mechanism behind memory functioning in the hippocampus (Bliss, 2003; Roman et al., 1987). The 1AKO mouse has been shown to have hippocampal-mediated learning and memory deficits (Klemenhagen et al., 2006c; Sarnyai et al., 2000). Furthermore, CRF₁ antagonism has been shown to reverse stress-induced learning and memory deficits in mice (Blank et al., 2003; Ivy et al., 2010). Given the increase in CRF-containing interneurons in the 1AKO, endogenous CRF within the hippocampus might contribute to the learning and memory deficits seen in these mice. This study showed that application of the CRF₁ antagonist CP-154526 before high-frequency stimulation
was able to rescue the LTP deficit in 1AKO mice. This points to a direct effect of the CRF peptide present within the hippocampus on CRF$_1$ receptors as the cause of the diminished LTP. One possible mechanism to explain this is that CRF is affecting another neurotransmitter system or receptor type. For example, the phosphorylation of AMPA receptors is known to enhance the induction of LTP, therefore CRF might be preventing this phosphorylation (Makino et al, 2011).

In a previous study from our lab, we looked at evoked AMPA receptor-mediated responses in 1AKO and WT cells. 1AKO pyramidal neurons demonstrated a clear deficit in AMPA response. Additionally, adult 1AKO mice also showed decreased amplitude of spontaneous EPSCs, demonstrating a decreased number of AMPA receptors. Results indicated that AMPA receptors may have been an underlying cause for the diminished LTP in the 1AKO. The current research attempted to replicate this study to see if the effect of the CRF$_1$ antagonist on LTP in the 1AKO is a direct result of changes in AMPA-mediated current. However, we were unable to reproduce the AMPAergic deficit. One possible reason behind this is that a change in animal facility occurred before this experiment was undertaken. Unfortunately there was construction occurring close to the new facility at the same time, and the mice may have been affected by this. Stressful surroundings, particularly during the critical period for 1AKO development, are known to have behavioral and electrophysiological effects, therefore more studies would be needed to determine accurate evoked responses (Ivy et al, 2010).

In summary, we demonstrated that hippocampal cytoarchitecture, synaptic plasticity, and CRF$_1$-mediated responses are altered in the 1AKO mouse. The exact
mechanisms underlying the rescue of LTP and the increased number of CRF-containing interneurons in CA1 will need to be researched further to gain a better understanding of the 1AKO as a genetic model of anxiety and cognitive dysfunction. Our results add to the existing body of data showing the impact of alterations in the 1AR.

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FIGURE LEGENDS

Figure 1: CRF-immunoreactive neurons in the 1AKO and WT hippocampal pyramidal cell layer. (A) A representative section showing DAB immunolabeling of CRF-containing cells (arrows). (B) Quantitative analysis of labeled cells revealed that the number of CRF-containing interneurons in the CA1 pyramidal cell layer per hippocampal slice was significantly higher in 1AKO (N=188 slices from 6 mice, p<0.0001) compared to WT (N=144 slices from 5 mice). Black lines indicate mean values.
Figure 1

A

B

Number of cells per slice

• WT
• 1AKO
Figure 2. Differential effects of CRF on 1AKO and WT pyramidal cells. (A) Adult WT (N=14) and 1AKO (N=13) pyramidal neurons show a membrane depolarization with application of CRF (left), but a decreased membrane resistance only in WT cells (right). (B) A main effect of genotype on the F-I plot showed 1AKO cells are more intrinsically excitable compared to WT cells. CRF had no effect on this measure. (C) 1AKO (N=14) and WT (N=20) cells at age P13 showed no differences in excitability before or after CRF. (D) P13 pyramidal cells showed a similar response to CRF compared to adult mice. Bath application of CRF resulted in a depolarization of cell membrane potential in both 1AKO and WT cells but a decrease in membrane resistance only in WT neurons. *p<0.05
Figure 2

A

B

C

D

Resting membrane potential (mV)

Resistance (MΩ)

Current (pA)

AP frequency (Hz)

Resting membrane potential (mV)

Resistance (MΩ)

Current (pA)

AP frequency (Hz)
Figure 3. The CRF<sub>1</sub> antagonist CP-154526 rescues the 1AKO deficit in LTP. (A) Representative fEPSP traces from WT and 1AKO slices averaged over a 30 min baseline (black) and for 60 min after tetanic stimulation (gray). Scale bar: 0.25mV, 5ms. (B) 1AKO slices (N=10) exhibited a deficit in LTP compared to those from WT mice (N=10). Arrow represents time of tetanus. (C) Representative fEPSP traces from WT and 1AKO slices in the presence of CP-154526 at baseline (black) and after tetanus (gray). (D) Application of 300nM CP-154526 beginning 30 min prior to tetanus resulted in LTP in 1AKO slices (N=9) that was equal to WT (N=9). There was no effect of CP-154526 on WT LTP.
Figure 3

A

WT

1AKO

B

\[ \text{fEPSP slope (% baseline)} \]

WT

1AKO

C

WT

1AKO

D

\[ \text{fEPSP slope (% baseline)} \]

WT CP-154526

1AKO CP-154526

Time (min)

0 10 20 30 40 50 60 70 80 90 100

75

100

125

150

175

200

225

250

275
Figure 4. CRF₁ blockade has no effect on AMPA receptor-mediated evoked EPSCs. (A) Representative raw data traces from WT and 1AKO neurons (top, scale bars = 200pA, 10ms). There was a significant deficit in AMPA-mediated eEPSC activity in 1AKO cells (N=15) compared to WT cells (N=12, p<0.0001) in a prior experiment from our lab (bottom). (B) This 1AKO deficit was not able to be reproduced in a second set of experiments (N=16 WT, 16 1AKO). 300nM CP-154526 had no effect on eEPSCs in either 1AKO or WT cells. *p<0.05
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